Journal of Virological Methods 239 (2017) 1–8

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

6 HCV genotyping 9G test and its comparison with VERSANT HCV genotype 2.0 assay (LiPA) for the hepatitis C virus genotyping

Wasun Chantratita[a,**], Keum-Soo Song[b], Choi GunHo[c], Viroj Pongthanapisith[a], Nipa Thongbaiphet[a], Garanyuta Wongtabtim[a], Ekawat Pasomsub[a], Kanokwan Angkanavin[a], Satish Balasaheb Nimse[b], Mukesh Digambar Sonawane[b], Shrikant Dasharath Warkad[b], Taisun Kim[b,*]

[a] Virology Laboratory, Department of Pathology, Faculty of Medicine, Ramathibodi Hospital, Mahidol University, Bangkok 10400, Thailand
[b] Institute for Applied Chemistry and Department of Chemistry, Hallym University, Chuncheon, 24252, Korea
[c] Biometrix Technology, Inc. 202 BioVenture Plaza, Chuncheon, 24232, Korea

Article history:
Received 13 October 2016
Received in revised form 21 October 2016
Accepted 23 October 2016
Available online 25 October 2016

Keywords:
Hcv
HCV genotyping
9G DNA technology
LiPA 2.0
Liver cirrhosis
Hepatocellular carcinoma

A B S T R A C T

In this article, we describe the 6 HCV Genotyping 9G test and its evaluation by using clinical samples and plasmid DNA standards. In tests with 981 plasmid DNA standards, the 6 HCV Genotyping 9G test showed higher than 92.5% sensitivity and 99.4% specificity. The 6 HCV Genotyping 9G test was compared with the VERSANT HCV Genotype 2.0 assay (LiPA 2.0) for detection and discrimination of HCV genotypes in clinical samples. The results of both tests were verified by genomic sequencing. The 6 HCV Genotyping 9G test demonstrated a 100% agreement with the sequencing results, which was higher than LiPA 2.0. These results indicate that the 6 HCV Genotyping 9G test can be a reliable, sensitive, and accurate diagnostic tool for the correct identification of HCV genotypes in clinical specimens. 6 HCV Genotyping 9G test can genotype six HCV types in 1 PCR in 30 min after PCR amplification. The 6 HCV Genotyping 9G test, thus provide critical information to physicians and assist them to apply accurate drug regimen for the effective hepatitis C treatment.

1. Introduction

Hepatitis C is a liver disease caused by the hepatitis C virus (HCV). Globally, 150 million people have chronic HCV infection. An estimated 700,000 people die each year from hepatitis C-related liver diseases including cirrhosis, hepatocellular carcinoma (HCC) and liver failure (WHO guidelines, 2016). The morbidity and mortality attributable to HCV infection continue to increase (Lavanchy, 2011). The treatment of HCV infection has become more complicated due to various genotypes and subtypes of HCV.

HCV is classified into seven genotypes, 67 confirmed and 20 provisional subtypes with distinct patterns of geographic distribution (Smith et al., 2014). Globally, HCV 1 is the most common (46.2%), followed by HCV 3 (30.1%), HCV 2 (9.1%), HCV 4 (8.3%), HCV 6 (5.4%), and HCV 5 (0.8%) (Messina et al., 2015). However, among Asian countries, the distribution and sub-typical composition of HCV6 are significantly different from overall global ratio. For example, HCV6a (24%) and HCV6n (39%) are predominant in Vietnam and Myanmar, respectively (Pham et al., 2011; Lwin et al., 2007). In Thai patients HCV6f is the most common HCV6 subtype (56%), followed by subtypes 6n (22%), and 6i (11%) (Akkarathamrongsin et al., 2010). A proper antiviral treatment can cure the HCV infection (Li and Lo, 2015). However, the knowledge about HCV genotype and subgenotype is crucial for the appropriate treatment (Wasitthankasem et al., 2015). It is essential to note that according to the recent recommendations by European Association for the Study of the Liver (EASL), the treatment of HCV infection significantly depends on particular HCV genotype or sub-genotype (EASL, 2015). The 2016 WHO guidelines for the screening, care, and treatment of patients with chronic HCV infection recommend the identification of HCV genotypes for the choice of the precise treatment regimen (WHO guidelines, 2016).

Currently, treatment of HCV has made significant advances with the help of drugs such as pegylated interferon (PegIFN)-α, ribavirin,

* Corresponding author.
** Corresponding author.
E-mail addresses: wasun.cha@mahidol.ac.th (W. Chantratita), hanlimsk@empas.com (K.-S. Song), ghchoi@bmtchip.com (C. GunHo), viroj.pon@mahidol.ac.th (V. Pongthanapisith), maymayenator@gmail.com (N. Thongbaiphet), garanyuta@gmail.com (G. Wongtabtim), ekawat.pas@mahidol.ac.th (E. Pasomsub), annvirus@yahoo.co.th (K. Angkanavin), satish_nimse@hallym.ac.kr (S.B. Nimse), mukeshsonawane87@hallym.ac.kr (M.D. Sonawane), shrikant.warkad@gmail.com (S.D. Warkad), tskim@hallym.ac.kr (T. Kim).

http://dx.doi.org/10.1016/j.jviromet.2016.10.009

and the direct-acting antivirals such as sofosbuvir, simeprevir, ledipasvir, ombitasvir, dasabuvir (Mishra et al., 2015). However, the choice of medicine or the combination of drugs and the duration of treatment are different for HCV genotypes 1a, 1b, 2, 3, 4, 5 and 6 (EASL 2015). Therefore, for accurate treatment of hepatitis C, it is imperative to detect and discriminate the crucial HCV genotypes such as 1a, 1b, 2, 3, 4, and 6 by using simple and accurate detection method.

Sequence analysis of specific regions such as NS5, core, E1, and 5′ untranslated region (5′UTR) is a gold standard for HCV genotyping (Firdaus et al., 2015). However, it is a time-consuming method, expensive, and requires highly trained professional to process the samples. Alternative nucleic acid tests such as real-time PCR (Hawkins et al., 1997; Beld et al., 2002), restriction fragment length polymorphism (Filippo et al., 2012), heteroduplex mobility analysis (White et al., 2000), and line-probe assay (Cai et al., 2013) are rapid HCV typing methods. The needs for specialized and expensive instruments have limited the applications of these methods in developing countries. The Versant HCV 2.0 assay (line probe assay [LiPA] 2.0), Abbott Realtime HCV Genotype II assay, and Trugene assay are widely used commercial assays for HCV genotyping. A recent report on the comparison of Trugene assay, LiPA 2.0, and sequencing indicate that these tests can fail to differentiate HCV subtypes 1a and 1b, which can lead to critical errors in clinical practice for correct use of directly acting antiviral agents (Chueca et al., 2016). Furthermore, the LiPA 2.0 assay and the Abbott Realtime HCV Genotype II assay have limitations in identifying HCV genotype 6 (Yang et al., 2014) and are expensive. Thus, for the implementation of a strict treatment regime in the management of hepatitis C, there is a need to find an inexpensive genotyping test that is rapid, simple, and precise.

In this study, 981 standard plasmid samples were used to evaluate the performance of 6 HCV Genotyping 9G test. All samples were tested under blinded codes. The performance of 6 HCV Genotyping 9G test was also evaluated by comparing it with the commercial VERSANT HCV Genotype 2.0 assay (LiPA 2.0) (Siemens Healthcare GmbH, Erlangen, Germany) and HCV sequencing for detection of six HCV genotypes 1a, 1b, 2, 3, 4, and 6 in the serum samples. The principle of 6 HCV Genotyping 9G test includes viral RNA isolation, complementary DNA (cDNA) synthesis, PCR amplification, and detection of PCR amplicons. The cDNA synthesis and PCR amplification are done simultaneously in single PCR tube by using RT-PCR. The 6 HCV Genotyping 9G test is a lateral flow strip membrane assay, wherein, the required 9G membranes are obtained by following the reported 9G technology (Song et al., 2013). The ssDNA oligonucleotide probes corresponding to the respective HCV genotypes are immobilized on the 9G membranes at specific positions. The immobilized probes hybridize with the complementary Cy5 labeled ssDNAs in the PCR product and allow the discrimination of six HCV genotypes. It is the first report of its kind to evaluate the performance of 6 HCV Genotyping 9G test and its comparison with the commercial HCV genotyping test. The 6 HCV Genotyping 9G test is designed to provide the necessary information to physicians by accurate detection of six HCV genotypes in 30 min after PCR. Thus, the 6 HCV Genotyping 9G test can enable doctors to select the most effective treatment regimen as per the EASL recommendations for hepatitis C management.

2. Materials and methods

2.1. Materials

All chemicals were purchased from Sigma-Aldrich Chemicals, Korea. The oligonucleotides were purchased from Bioneer, Korea. Glass fiber membrane (2.5 × 7.5 cm) was obtained from Whatman, Springfield, UK. All washing solvents for the substrates are of HPLC grade from SK Chemicals, Korea. Ultrapure water (18 M Ω/cm) was obtained from a Milli-Q purification system (Millipore). Oligonucleotides were lined using dispenser (BioDot Technologies, Inc., 2852 Alton Pkwy, Irvine, CA 92606, USA). Hybridization was done at 25 °C. The fluorescence intensities were recorded on the BMT Reader™, Biometrix Technology Inc., South Korea. VERSANT HCV Genotype 2.0 assay (LiPA 2.0) (Siemens Healthcare GmbH, Erlangen, Germany) kit was used as a standard commercial product.

2.2. Composition of different solutions used for 6 HCV genotyping 9G test

a) Hybridization buffers (pH = 7.4): 20% Formamide, 0.25% Triton X-100, 6x SSC; b) Washing buffer solution (pH = 7.4): 20% Formamide, 0.25% Triton X-100, 6x SSC.

2.3. Standard DNA samples

The cDNA standard samples (plasmid DNAs) containing corresponding 300-bp sequences of the 5′UTR of HCV RNA were obtained from Bioneer Inc., Daejeon, Korea. The plasmid cDNA sequences were unique to the HCV genotypes 1a, 1b, 2, 3, 4, 6a, 6f, 6i, and 6n. Plasmid standard samples (n = 981) were used for the test under the blinded codes. All standard samples were diluted with Tris-elution buffer utilized in the regular extraction kits used for clinical specimens. Thus, the process of evaluation by using clinical samples was mimicked in the 6 HCV Genotyping 9G test with plasmid cDNA standard samples. The 981 samples were separated in eight different categories as 1×10^6 copies/test (7.5×10^6 copies/mL), 1000 copies/test (7.5×10^3 copies/mL), 50 copies/test (375 copies/mL), 10 copies/test (75 copies/mL), 5 copies/test (38 copies/mL), 2.5 copies/test (19 copies/mL), 1 copy/test (7.5 copies/mL), and 0 copy/test (negative samples). Table 1 depicts the distribution of 981 standard samples corresponding to the HCV genotypes 1a, 1b, 2, 3, 4, 6a, 6f, 6i, and 6n in eight concentration categories. All samples were tested under blinded codes. PCR was performed on plasmid cDNA samples by

Table 1
Concentration dependent distribution of standard samples used in 6 HCV Genotyping 9G Test.

Copies/test	HCV genotypes										Total
	1a	1b	2	3	4	6a	6f	6i	6n	NC	
1×10^6	5	5	5	5	5	5	5	5	5	9	54
1000	5	5	5	5	5	5	5	5	5	9	54
50	10	10	10	10	10	10	10	10	10	9	99
10	10	10	10	10	10	10	10	10	10	9	99
5	24	24	24	24	24	24	24	24	24	9	225
2.5	24	24	24	24	24	24	24	24	24	9	225
1	24	24	24	24	24	24	24	24	24	9	225
Total	102	102	102	102	102	102	102	102	102	63	981

NC: negative control samples containing no HCV standard DNA.

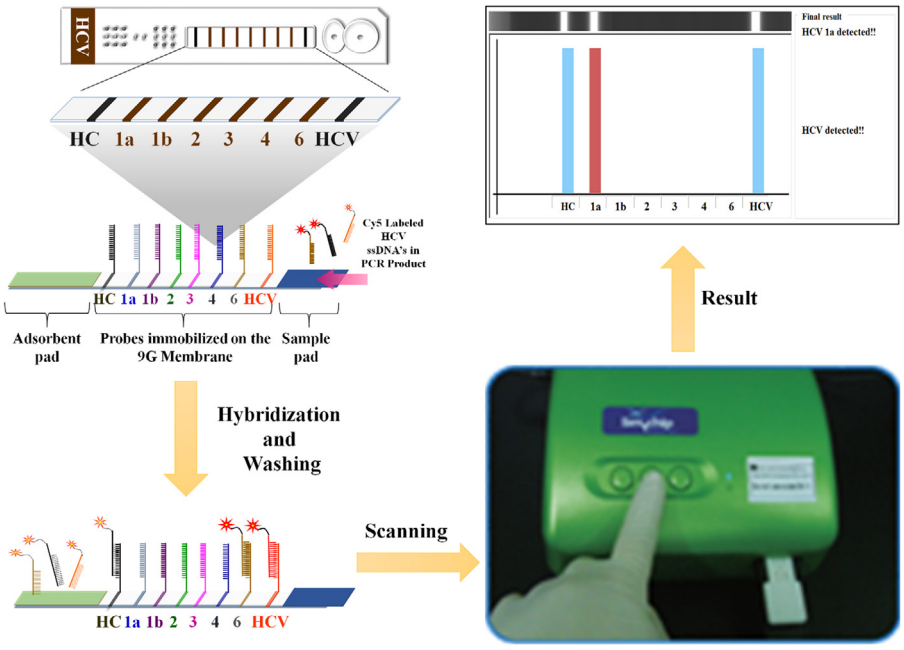

Fig. 1. Position of the respective probes on 9G membrane and the experimental protocol of 6 HCV Genotyping 9G Test.

following the process used in the 6 HCV Genotyping 9G test for clinical samples.

2.4. Clinical samples

A total of 98 patients serum samples were used in this study. 63/98 samples were from patients known to be infected with the HCV. In the group of 98 serum samples, 35 were from HCV negative patients. All samples had been previously genotyped by LiPA 2.0 according to the manufacturer's instructions. The HCV RNA extraction was performed on the NucliSENS easyMAG (bioMérieux, Boxtel, Netherlands), which automatically extract the nucleic acids from the clinical samples. 200 μL of plasma was added to the lysis buffer followed by 10 min incubation at room temperature. Magnetic silica particles were used for nucleic acid binding for 10 min at room temperature. Silica particles were washed with different buffers. HCV RNA was eluted in 50 μL of Tris-elution buffer and subsequently transcribed into cDNA by following the protocols of LiPA 2.0 and 6 HCV Genotyping 9G test.

2.5. HCV genotyping 9G test

The principle of 6 HCV Genotyping 9G test includes viral RNA isolation, complementary DNA (cDNA) synthesis, PCR amplification, and detection of PCR amplicons. The primers in the 6 HCV Genotyping 9G test amplify the 5′UTR for genotyping of six HCV genotypes. The probes allow the discrimination of HCV genotypes 1a, 1b, 2, 3, 4, 6a or 6f, and 6i or 6n at 25 °C in less than 30 min after PCR.

As shown in Fig. 1, the HC (hybridization control) probe, HCV (probe specific for the detection of HCV) probe, and six other probes specific to the HCV genotypes 1a, 1b, 2, 3, 4, and 6, were immobilized on the glass fiber membranes to produce the 9G membranes according to the recently reported method (Song et al., 2013). All probes were selected according to the generalized probe selection method (Nimse et al., 2011). Fig. 1 also depicts the simple experimental protocol of 6 HCV Genotyping 9G test to obtain final results.

The HC probe serves as an internal standard for hybridization control. The HCV probe is used to detect the presence or absence of HCV in the sample. As shown in Fig. 2, when 6 HCV Genotyping 9G test shows three signals corresponding to probes including the HC, HCV, and 1a or 2 or 3 or 4, a sample is designated as an HCV 1a or 2 or 3 or 4, respectively. The sample is identified as HCV 1b if the test shows four signals for probes including HC, HCV, 1a, and 1b, respectively.

When the test shows three signals corresponding to the probes HC, HCV, and 6, the sample is genotyped as HCV 6a or 6f. Whereas, if the test shows four signals corresponding to the probes HC, HCV, 1a, and 6, the sample is genotyped as HCV 6i or 6n. When, 6 HCV Genotyping 9G test shows two signals corresponding to the HC and HCV probes, the sample is identified to contain the HCV genotype other than the HCV 1a, 1b, 2, 3, 4, 6a, 6f, 6i, and 6n. However, for an HCV-negative sample, the test shows only one signal corresponding to the HC probe.

The process of 6 HCV Genotyping 9G test after completion of cDNA synthesis followed by PCR amplification is briefly discussed here. 110 μL of hybridization solution was added to each PCR tube containing 20 μL PCR product. Then, 110 μL of this mixture was loaded into the sample port of the 6 HCV Genotyping 9G test and allowed to stand for 20 min at 25 °C. After 20 min, 200 μL of washing solution was loaded into the washing port of the 6 HCV Genotyping 9G test and allowed to stand for 8 min at 25 °C. After 8 min, each 6 HCV Genotyping 9G test strips was scanned on the BMT Reader™, (Biometrix Technology Inc. Chuncheon, South Korea) to obtain the final results as shown in Fig. 2. Table 2 and Table 3 summarize the results of the 6 HCV Genotyping 9G test on standard samples. Table 4 and Table 5 summarize the comparative results on clinical samples obtained by 6 HCV Genotyping 9G test, LiPA 2.0, and sequencing.

2.6. LiPA 2.0

LiPA 2.0 is a reverse hybridization line probe assay. Viral RNA was purified using a QIAamp viral RNA minikit (Qiagen, Hilden, Germany) and was subjected to reverse transcription-PCR (RT-PCR) with a Versant HCV amplification 2.0 kit (manufactured by Innogenetics, Ghent, Belgium, for Siemens, Tarrytown, NY, USA). The 240-bp 5′UTR and 270-bp core fragments were co-amplified to

Table 2
Results of 6 HCV Genotyping 9G Test on 981 standard samples at different concentrations.

Copies/test	Variant	TP	TN	FP	FN	Total	% Sensitivity (95% CI)	% Specificity (95% CI)
1×10^6	HCV±	45	9	0	0	54	100.0 (92.1–100.0)	100.0 (66.3–100.0)
	1a	5	49	0	0	54	100.0 (47.8–100.0)	100.0 (92.8–100.0)
	1b	5	49	0	0	54	100.0 (47.8–100.0)	100.0 (92.8–100.0)
	2	5	49	0	0	54	100.0 (47.8–100.0)	100.0 (92.8–100.0)
	3	5	49	0	0	54	100.0 (47.8–100.0)	100.0 (92.8–100.0)
	4	5	49	0	0	54	100.0 (47.8–100.0)	100.0 (92.8–100.0)
	6a	5	49	0	0	54	100.0 (47.8–100.0)	100.0 (92.8–100.0)
	6f	5	49	0	0	54	100.0 (47.8–100.0)	100.0 (92.8–100.0)
	6i	5	49	0	0	54	100.0 (47.8–100.0)	100.0 (92.8–100.0)
	6n	5	49	0	0	54	100.0 (47.8–100.0)	100.0 (92.8–100.0)
1000	HCV±	45	9	0	0	54	100.0 (92.1–100.0)	100.0 (66.3–100.0)
	1a	5	49	0	0	54	100.0 (47.8–100.0)	100.0 (92.8–100.0)
	1b	5	49	0	0	54	100.0 (47.8–100.0)	100.0 (92.8–100.0)
	2	5	49	0	0	54	100.0 (47.8–100.0)	100.0 (92.8–100.0)
	3	5	49	0	0	54	100.0 (47.8–100.0)	100.0 (92.8–100.0)
	4	5	49	0	0	54	100.0 (47.8–100.0)	100.0 (92.8–100.0)
	6a	5	49	0	0	54	100.0 (47.8–100.0)	100.0 (92.8–100.0)
	6f	5	49	0	0	54	100.0 (47.8–100.0)	100.0 (92.8–100.0)
	6i	5	49	0	0	54	100.0 (47.8–100.0)	100.0 (92.8–100.0)
	6n	5	49	0	0	54	100.0 (47.8–100.0)	100.0 (92.8–100.0)
50	HCV±	90	9	0	0	99	100.0 (96.0–100.0)	100.0 (66.4–100.0)
	1a	10	88	1	0	99	100.0 (69.2–100.0)	98.9 (93.2–100.0)
	1b	10	89	0	0	99	100.0 (69.2–100.0)	100.0 (95.9–100.0)
	2	10	89	0	0	99	100.0 (69.2–100.0)	100.0 (95.9–100.0)
	3	10	89	0	0	99	100.0 (69.2–100.0)	100.0 (95.9–100.0)
	4	10	89	0	0	99	100.0 (69.2–100.0)	100.0 (95.9–100.0)
	6a	10	89	0	0	99	100.0 (69.2–100.0)	100.0 (95.9–100.0)
	6f	10	89	0	0	99	100.0 (69.2–100.0)	100.0 (95.9–100.0)
	6i	10	89	0	0	99	100.0 (69.2–100.0)	100.0 (95.9–100.0)
	6n	10	89	0	0	99	100.0 (69.2–100.0)	100.0 (95.9–100.0)
10	HCV±	90	9	0	0	99	100.0 (96.0–100.0)	100.0 (66.4–100.0)
	1a	10	88	1	0	99	100.0 (69.2–100.0)	98.8 (93.2–100.0)
	1b	10	89	0	0	99	100.0 (69.2–100.0)	100.0 (95.9–100.0)
	2	10	89	0	0	99	100.0 (69.2–100.0)	100.0 (95.9–100.0)
	3	10	89	0	0	99	100.0 (69.2–100.0)	100.0 (95.9–100.0)
	4	10	89	0	0	99	100.0 (69.2–100.0)	100.0 (95.9–100.0)
	6a	10	89	0	0	99	100.0 (69.2–100.0)	100.0 (95.9–100.0)
	6f	10	88	1	0	99	100.0 (69.2–100.0)	98.8 (93.2–100.0)
	6i	10	89	0	0	99	100.0 (69.2–100.0)	100.0 (95.9–100.0)
	6n	10	89	0	0	99	100.0 (69.2–100.0)	100.0 (95.9–100.0)
5	HCV±	210	9	0	6	225	97.2 (94.1–98.9)	100.0 (66.4–100.0)
	1a	24	200	1	0	225	100.0 (85.7–100.0)	99.5 (97.3–100.0)
	1b	21	200	1	3	225	87.5 (67.6–97.3)	99.5 (97.2–100.0)
	2	24	201	0	0	225	100.0 (85.8–100.0)	100.0 (98.2–100.0)
	3	22	201	0	2	225	91.7 (73.0–98.9)	100.0 (98.2–100.0)
	4	20	199	2	4	225	83.3 (62.6–95.3)	99.0 (96.5–99.8)
	6a	21	201	0	3	225	87.5 (67.6–97.3)	100.0 (98.1–100.0)
	6f	22	201	0	2	225	91.7 (73.0–98.9)	100.0 (98.2–100.0)
	6i	20	201	0	4	225	83.3 (62.6–95.2)	100.0 (98.1–100.0)
	6n	22	199	2	2	225	91.6 (73.0–98.9)	99.0 (96.5–99.8)
2.5	HCV±	178	9	0	38	225	82.4 (76.7–87.2)	77.8(39.9–97.1)
	1a	18	201	0	6	225	75.0 (53.3–90.2)	100.0 (98.2–100.0)
	1b	16	201	0	8	225	66.6 (44.6–84.3)	100.0 (98.2–100.0)
	2	15	197	4	9	225	62.5 (40.5–81.2)	98.0 (94.9–99.4)
	3	16	199	2	8	225	66.6 (44.6–84.3)	99.0 (96.4–99.8)
	4	13	200	1	11	225	54.1 (32.8–74.4)	99.5 (97.2–99.9)
	6a	14	201	0	10	225	58.3 (36.6–77.8)	100.0 (98.1–100.0)
	6f	13	201	0	11	225	54.2 (32.8–74.4)	100.0 (98.1–100.0)
	6i	14	197	4	10	225	58.3 (36.6–77.8)	98.0 (94.9–99.4)
	6n	14	199	2	10	225	58.3 (36.6–77.8)	99.0 (96.4–99.8)
1	HCV±	166	9	0	50	225	76.9 (70.7–82.3)	100.0 (66.4–100.0)
	1a	13	200	1	11	225	54.1 (32.8–74.4)	99.5 (97.2–99.9)
	1b	10	198	3	14	225	41.6 (22.1–63.3)	98.5 (95.7–99.6)
	2	12	199	2	12	225	50.0 (29.1–70.8)	99.0 (96.4–99.8)
	3	10	201	0	14	225	41.6 (22.1–63.3)	100.0 (98.2–100.0)
	4	13	201	0	11	225	54.1 (32.8–74.4)	100.0 (98.1–100.0)
	6a	4	200	1	20	225	16.6 (4.7–37.3)	99.5 (97.2–99.9)
	6f	5	200	1	19	225	20.8 (7.1–42.1)	99.5 (97.2–99.9)
	6i	6	199	2	18	225	25.0 (9.7–46.7)	99.0 (96.4–99.8)
	6n	5	200	1	19	225	20.8 (7.1–42.1)	99.5 (97.2–99.9)

TP: true positive, TN: true negative, FP: false positive, FN: false negative.

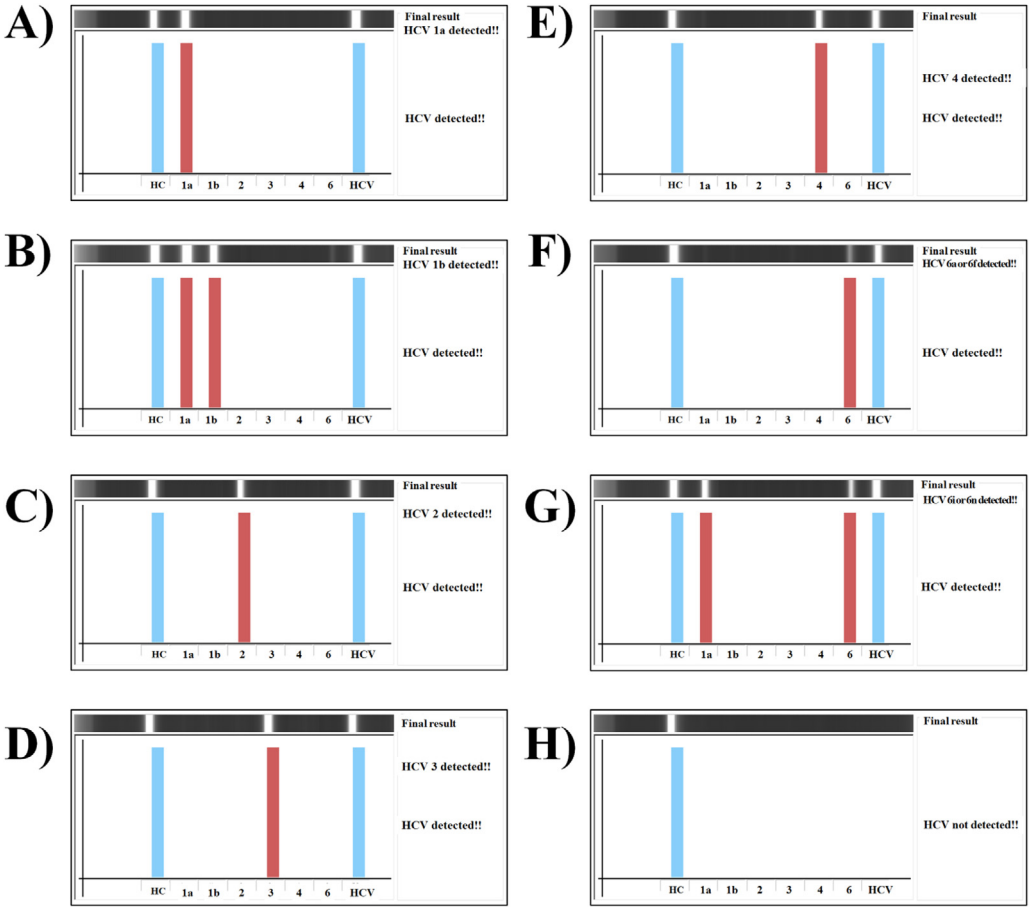

Fig. 2. The final result for HCV genotypes A) 1a, B) 1b, C) 2, D) 3, E) 4, F) 6a or 6f, G) 6i or 6n, and H) HCV negative samples in 6 HCV Genotyping 9G Test.

Table 3
Overall sensitivity and specificity of the 6 HCV Genotyping 9G Test on 531 standard samples with concentrations of 5 copies/test ~ 1×10^6 copies/test.

Variant	TP	TN	FP	FN	% Sensitivity (95% CI)	% Specificity (95% CI)
HCV±	480	45	0	6	98.7 (97.3–99.5)	100.0 (92.1–100.0)
1a	54	474	3	0	100.0 (93.4–100.0)	99.4 (98.2–99.8)
1b	51	476	1	3	94.4 (84.6–98.8)	99.8 (98.8–99.9)
2	54	477	0	0	100.0 (93.4–100.0)	100.0 (99.2–100.0)
3	52	477	0	2	96.3 (87.2–99.5)	100.0 (99.2–100.0)
4	50	475	2	4	92.5 (82.1–97.9)	99.5 (98.4–99.9)
6a	51	477	0	3	94.4 (84.6–98.8)	100.0 (99.2–100.0)
6f	52	476	1	2	96.3 (87.2–99.5)	99.7 (98.8–99.9)
6i	50	477	0	4	92.6 (82.1–97.9)	100.0 (99.2–100.0)
6n	52	475	2	2	96.3 (87.3–99.5)	99.5 (98.4–99.9)

Table 4
Results of 6 HCV Genotyping 9G Test and LiPA 2.0 assay for 79 samples.

Results of LiPA 2.0	Results of 6 HCV Genotyping 9G Test								
	1a	1b	2	3	4	6a or 6f	6i or 6n	Negative	Total
1a	5								5
1b		15							15
2			0						0
3				13					13
4					0				0
						4	7		
6 (c-l)						11			11
Negative							35	35	
Total	5	15	0	13	0	4	7	35	79

produce biotinylated PCR product. For the detection of HCV genotypes, a biotinylated PCR product is allowed to hybridize with the immobilized oligonucleotide probes that are specific for the 5′-UTRs and core regions of the six HCV genotypes. The hybridized biotinylated DNA PCR product is detected by using the signal amplification technique, which uses alkaline phosphatase-labeled

Table 5
Comparison of 6 HCV Genotyping 9G Test and LiPA 2.0 with the sequencing for 19 discordant samples (HCV genotype is given in parenthesis).

HCV Genotype	Sequencing	6 HCV Genotyping 9G Test	LiPA 2.0
1a	0	0	0
1b	11	11 (1b)	2 (1a), 9 (6(c-l))
2	0	0	0
3	1	1 (3)	1 (6(c-l))
4	0	0	0
6f	0	0	0
6i	7	7 (6i or 6n)	5 (1b), 2 (1a)
Negative	0	0	0
Total	19	19	19

streptavidin and 5-bromo-4-chloro-3-indolylphosphate (BCIP)-*p*-nitroblue tetrazolium chromogen. The results were interpreted according to the LiPA 2.0 interpretation chart, where the line patterns and the corresponding genotyping results are listed. Tables 4 and **5** show the results of the LiPA 2.0 assay in comparison with 6 HCV Genotyping 9G test and sequencing.

2.7. Sequence analysis

The sequence of amplified fragment (from clinical samples) was determined on Applied Biosystems (ABI) 3730 × L DNA analyzer (Life Technologies Co., Carlsbad, CA, USA). The specific HCV genotypes were confirmed by comparing the obtained sequences with the reported sequences on the Basic Local Alignment Search Tool (BLAST) database of NCBI. The results of the sequencing analysis are shown in Tables 4 and 5.

3. Results

3.1. Standard samples

As shown in Tables 1 and 2, 981-blinded samples were tested to evaluate the 6 HCV Genotyping 9G test for the accurate detection and genotyping of HCV strains. The PCR amplification to obtain the HCV DNA amplicons was performed on each of the 981 samples. Out of 981 samples, 54 samples each was in the concentration category of 1×10^6 copies/test and 1000 copies/test. Out of these 54 samples, nine samples were negative and remaining 45 samples were further divided into five samples each of HCV genotypes 1a, 1b, 2, 3, 4, 6a, 6f, 6i, and 6n, respectively. Similarly, there were 99 samples each in the concentration category of 50 copies/test and 10 copies/test. Out of these 99 samples, nine samples were negative, and remaining 90 samples were further divided into ten samples each of HCV genotypes 1a, 1b, 2, 3, 4, 6a, 6f, 6i, and 6n, respectively. There were 225 samples each in the concentration category of 5 copies/test and 2.5 copies/test and 1 copy/test. Out of these 225 samples, nine samples were negative, and remaining 116 samples were further divided into 24 samples each of HCV genotypes 1a, 1b, 2, 3, 4, 6a, 6f, 6i, and 6n, respectively. Hence, out of 981 samples, 63 samples were true HCV negative and 918 samples were true HCV positive.

The data presented in Table 2 shows the sensitivity and specificity of each probe at different concentrations of HCV cDNA in the samples. The 6 HCV Genotyping 9G test correctly detected 918 samples as true positive (with HCV cDNA) and 63 samples as true negative (without HCV cDNA) on the HCV line irrespective of the concentration of a cDNA in the sample. The HCV probe showed almost 100% sensitivity and specificity, respectively. However, as shown in Table 2, the sensitivity of the probes corresponding to the HCV genotypes 1a, 1b, 2, 3, 4, 6a, were significantly lower for the samples containing HCV cDNA with concentrations of 2.5 copies/test (54.1–82.4%) and 1 copy/test (16.6–76.9%). For these samples, the sensitivity of the six probes were in the range of 20–75%. However, the samples containing 5 copies/test of HCV cDNA all probes demonstrated the sensitivity and specificity of 83–100% and 99–100%, respectively. Therefore, the limit of detection (LOD) of the 6 HCV Genotyping 9G test for the detection and discrimination of HCV genotypes 1a, 1b, 2, 3, 4, 6a, 6f, 6i, and 6n were decided to be 5 copies/test (38 copies/mL). Thus, the LOD of the 6 HCV Genotyping 9G test is much lower than the LOD (4810 IU/mL) reported for the commercial assays (Nadarajah et al., 2007).

The LOD being set to 5 copies/test, the data on the 531 standard plasmid samples was used for the determination of the overall sensitivity and specificity of the 6 HCV Genotyping 9G test as shown in Table 3. Out of 531 samples, 486 samples (54 samples per HCV genotypes 1a, 1b, 2, 3, 4, 6a, 6f, 6i, and 6n) were true HCV positive and 45 samples were true HCV negative. All samples were correctly detected by each probe corresponding to the HCV genotypes 1a, 1b, 2, 3, 4, 6a, 6f, 6i, and 6n, respectively. The probe corresponding to the HCV genotype 1a successfully identified the HCV 1a in all 54 HCV true positive samples. However, 3/477 samples that did not have any HCV 1a cDNA in them were falsely detected as HCV 1a, false positive results. Out or 54 true positive samples and 477 true negative samples for HCV 1b, the probe for HCV 1b showed one false positive result and three false negative result, respectively. Out of 54 true positive samples for each of the HCV type 2, 3, 4, and 6a, 6f, 6i, and 6n, the probes corresponding to respective HCV genotypes demonstrated 0, 2, 4, 3, 2, 4, and 2 false negative results, respectively. However, the false negative rate for probes corresponding to the HCV type 2, 3, 4, and 6a, 6f, 6i, and 6n was low.

The data presented in Table 3 shows the sensitivity and specificity of each probe used in the 6 HCV Genotyping 9G test for the detection of HCV genotypes in the standard plasmid DNA samples. The sensitivity of all six probes for detection of HCV genotypes in the standard samples were in the range 92.6-100%. The specificity of all six probes was higher than 99%. The sensitivity and specificity of the probes were in the range 83.3-100% and 77.8%-100%, respectively, for samples containing more than 5 copies/test of HCV cDNA. These results indicate that 6 HCV Genotyping 9G test can be used effectively for the detection and genotyping of the HCV in clinical samples.

3.2. Clinical samples

As shown in Table 4, 6 HCV Genotyping 9G test and LiPA 2.0 assay showed concordance in the detection of HCV genotypes in 79/98 clinical samples and discordance in the detection of 19/98 samples. Both tests showed 80.6% agreement and 19.4% discordance in the results for the detection of HCV genotypes. As shown in Table 4, both tests clearly identified 35 HCV negative samples as HCV negative.

Out of 44 HCV positive samples with the concordance of results in both tests, five (11.4%), 15 (34.1%), 13 (29.5%), and 11 (25.0%) samples, were HCV genotypes 1a, 1b, 3, and 6, respectively. LiPA 2.0 assay identified eleven HCV6 genotype samples as HCV 6(c-l). Whereas, 6 HCV Genotyping 9G test identified 4 (9.1%) and 7 (15.9%)

out 11 HCV6 genotype samples as HCV(6a or 6f) and HCV(6i or 6n), respectively. The 6 HCV Genotyping 9G test can subtype the HCV6 genotype into two categories viz. HCV(6a or 6f) and HCV(6i or 6n).

The 19/98 (19.4%) samples that showed discordance in the results of 6 HCV Genotyping 9G test and LiPA 2.0 assay were genotyped by sequencing. Table 4 summarizes the comparison of the results of sequencing, 6 HCV Genotyping 9G test and LiPA 2.0 assay. Sequencing identified 11 (57.9%), 1 (5.3%), and 7 (36.8%) samples out of 19 samples as HCV genotypes 1b, 3, and 6i, respectively. As shown in Table 5, 6 HCV Genotyping 9G test showed 100% agreement with the results of sequencing for the detection of HCV1b, HCV3, and HCV6i samples. However, LiPA 2.0 assay showed complete disagreement with sequencing analysis. The eleven HCV1b samples were correctly identified as HCV1b in 6 HCV Genotyping 9G test. However, of the eleven HCV1b samples LiPA 2.0 assay falsely detected two and nine samples as HCV1a and HCV6 (c-l), respectively. An HCV3 sample was designated as HCV6 (c-l) in the LiPA 2.0 assay. All of the seven HCV6i samples were detected as HCV(6i or 6n) in the 6 HCV Genotyping 9G test. However, out seven HCV6i samples, LiPA 2.0 assay detected 5 and 2 samples as HCV1b and HCV1a, respectively.

These results clearly indicate that, 6 HCV Genotyping 9G test is superior over LiPA 2.0 assay for the genotyping of HCV. The overall sensitivity and specificity of 6 HCV Genotyping 9G test were 100.0% (94.3–100.0% at 95% CI) and 100.0% (90.0–100.0% at 95% CI), respectively. At the individual subtype level, both the sensitivity and specificity of the 6 HCV Genotyping 9G test were 100%. It was observed in this test that the LiPA 2.0 assay has limitations in the correct detection and discrimination of the HCV genotypes 1a, 1b, and 6. The overall sensitivity and specificity of LiPA 2.0 assay were 69.8% (56.9–80.7% at 95% CI) and 100.0% (90.0–100.07% at 95% CI), respectively.

4. Discussion

The evaluation of 6 HCV Genotyping 9G test for HCV genotyping and subtyping was conducted on 981 blinded standard samples, with results indicating more than 92% sensitivity and 99% specificity for the detection of HCV genotypes. The results of the 6 HCV Genotyping 9G test on the standard samples clearly indicate that this test can discriminate the HCV 1a, 1b, 2, 3, 4, and HCV 6 (6a or 6f, and 6i or 6n). Essentially, the application of any new test can be adjudicated based on its performance for the detection of HCV genotypes in the clinical samples. Thus, 98 clinical samples were used in the preliminary clinical evaluation of the 6 HCV Genotyping 9G test.

In this study, the commercial LiPA 2.0 assay and sequencing analysis were used as the standards for the detection of the HCV genotypes in the 98 patients serum samples under blinded codes. The 6 HCV Genotyping 9G test showed 100.0% concordance with sequence analysis in the 19 samples. However, the LiPA 2.0 assay showed complete discordance with sequence results for the 19 samples that were discordant with 6 HCV Genotyping 9G test.

The limitation of the LiPA 2.0 assay for the correct identification of HCV 1a, 1b, and HCV 6 is reported (Chueca et al., 2016). It was observed in this study that the HCV genotypes 1a, 1b, 2, 3, 4, and 6 along with their subtypes have more than 95% sequence homology. Thus, the high percentage of sequence homology is a likely reason for the discrepancies in the results of LiPA 2.0 assay.

Sequence analysis is considered the gold standard and it is observed in this study that the results of 6 HCV Genotyping 9G test were in 100% agreement with it. It is important to note that according to an earlier report, the 9G membrane technology used in the 6 HCV Genotyping 9G test enables detection of genomic DNAs with a concentration of 38 copies/mL. Moreover, the sensitivity and specificity of 6 HCV Genotyping 9G test for the detection of the HCV genotype viz. 1a, 1b, 2, 3, 4, and 6 (6a or 6f and 6a or 6i) in the standard plasmid samples with the concentration higher than 38 copies/mL was over 94% and 99%, respectively. At higher concentrations the sensitivity and specificity of the 6 HCV Genotyping 9G test is 100.0%.

The correct identification of the HCV genotypes in the HCV-infected patients is critical information for physicians to apply the proper drug regimen. Therefore, it is vital for the clinical laboratories to use the most accurate method to provide in-depth information on HCV infection to clinicians for better patient care. It is important to note that 6 HCV Genotyping 9G test uses single RT-PCR for the identification of HCV genotypes and delivers final results in less than 30 min at 25 °C after PCR.

5. Conclusion

The evaluation of 6 HCV Genotyping 9G test by using 981 standard samples showed the overall sensitivity and specificity to be higher than 92.5% and 99.4%, respectively. The results of evaluation of the 6 HCV Genotyping 9G test indicates that it is a reliable, sensitive, and accurate diagnostic tool for the detection and genotyping HCV genotypes in clinical samples. However, only 15 clinical samples were used in this study. Therefore, the evaluation of the 6 HCV Genotyping 9G test with a broader range of clinical samples would be necessary to substantiate the findings in this report. The 6 HCV Genotyping 9G test provides valuable information to physicians by detecting and discrimination the six HCV genotypes in 30 min after PCR, which is vital information for the choice of definitive drug therapy.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgments

This research was supported by the Ministry of Trade, Industry & Energy (MOTIE), Korea Institute for Advancement of Technology (KIAT) through the Encouragement Program for The Industries of Economic Cooperation Region (Project No.: R0004191). This work was also supported, in part, by Center for medical genomics, Ramathibodi Hospital, Mahidol University, Bangkok, Thailand.

References

Akkarathamrongsin, S., Praianantathavorn, K., Hacharoen, N., Theamboonlers, A., Tangkijvanich, P., Tanaka, Y., Mizokami, M., Poovorawan, Y., 2010. Geographic distribution of hepatitis C virus genotype 6 subtypes in Thailand. J. Med. Virol. 82, 257–262.

Beld, M., Sentjens, R., Rebers, S., Weegink, C., Weel, J., Sol, C., Boom, R., 2002. Performance of the New Bayer VERSANT HCV RNA 3.0 assay for quantitation of hepatitis C virus RNA in plasma and serum: conversion to international units and comparison with the Roche COBAS Amplicor HCV Monitor Version 2.0, assay. J. Clin. Microbiol. 40, 788–793.

Cai, Q., Zhao, Z., Liu, Y., Shao, X., Gao, Z., 2013. Comparison of three different HCV genotyping methods: core, NS5B sequence analysis and line probe assay. Int. J. Mol. Med. 31 (2), 347–352.

Chueca, N., Rivadulla, I., Lovatti, R., Reina, G., Blanco, A., Fernandez-Caballero, J.A., Cardeñoso, L., Rodriguez-Granjer, J., Fernandez-Alonso, M., Aguilera, A., Alvarez, M., Galán, J.C., García, F., 2016. Using NS5B sequencing for hepatitis C virus genotyping reveals discordances with commercial platforms. PLoS One 11 (4), e0153754.

European Association for Study of Liver, 2015. EASL recommendations on treatment of hepatitis C. J. Hepatol. 63, 199–236.

Filippo, D., Cortes-Mancera, F., Beltran, M., Patricia Arbelaez, M., Jaramillo, S., Restrepo, J.C., Correa, G., Navas, M., 2012. Molecular characterization of hepatitis c virus in multi-transfused Colombian patients. Virol. J. 9 (242), 1–8.

Firdaus, R., Saha, K., Biswas, A., Sadhukhan, P.C., 2015. Current molecular methods for the detection of hepatitis C virus in high risk group population: a systematic review. World J. Virol. 4 (1), 25–32.

Hawkins, A., Davidson, F., Simmonds, P., 1997. Comparison of plasma virus loads among individuals infected with hepatitis C virus (HCV) genotypes 1, 2, and 3 by quantiplex HCV RNA assay versions 1 and 2, Roche Monitor assay, and an in-house limiting dilution method. J. Clin. Microbiol. 35, 187–192.
Lavanchy, D., 2011. Evolving epidemiology of hepatitis C virus. Clin. Microbiol. Infect. 17, 107–115.
Li, H., Lo, S., 2015. Hepatitis C virus: virology, diagnosis and treatment. World J. Hepatol. 7 (10), 1377–1389.
Lwin, A.A., Shinji, T., Khin, M., Win, N., Obika, M., Okada, S., Koide, N., 2007. Hepatitis C virus genotype distribution in Myanmar: predominance of genotype 6 and existence of new genotype 6 subtype. Hepatol. Res. 37, 337–345.
Messina, J.P., Humphreys, I., Flaxman, A., Brown, A., Cooke, G.S., Pybus, O.G., Barnes, E., 2015. Global distribution and prevalence of hepatitis C virus genotypes. Hepatology 61, 77–87.
Mishra, P., Murray, J., Birnkrant, D., 2015. Direct-acting antiviral drug approvals for treatment of chronic hepatitis C virus infection: scientific and regulatory approaches to clinical trial designs. Hepatology 62 (4), 1298–1303.
Nadarajah, R., Khan, G.Y., Miller, S.A., Brooks, G.F., 2007. Evaluation of a new-generation line-probe assay that detects 5' untranslated and core regions to genotype and subtype hepatitis C virus. Am. J. Clin Pathol. 128 (2), 300–304.
Nimse, S.B., Song, K., Kim, J., Kim, H., Nguyen, V., Ta, V., Kim, T., 2011. Generalized probe selection method for DNA Chips. Chem. Commun. 47, 12444–12446.
Pham, V.H., Nguyen, H.D., Ho, P.T., Banh, D.V., Pham, H.L., Pham, P.H., Lu, L., Abe, K., 2011. Very high prevalence of hepatitis C virus genotype 6 variants in southern Vietnam: large-scale survey based on sequence determination. Jpn. J. Infect. Dis. 64, 537–539.
Smith, D.B., Bukh, J., Kuiken, C., Muerhoff, A.S., Rice, C.M., Stapleton, J.T., Simmonds, P., 2014. Expanded classification of hepatitis C virus into 7 genotypes and 67 subtypes: updated criteria and genotype assignment web resource. Hepatology 59, 318–327.
Song, K., Nimse, S.B., Kim, J., Sayyed, D.R., Kim, T., 2013. New platform for convenient genotyping system. Chem. Commun. 49, 2661–2663.
WHO guidelines, 2016. http://www.who.int/hepatitis/publications/hepatitis-c-guidelines-2016/en/.
Wasitthankasem, R., Vongpunsawad, S., Siripon, N., Suya, C., Chulothok, P., Chaiear, K., Rujirojindakul, P., Kanjana, S., Theamboonlers, A., Tangkijvanich, P., Poovorawan, Y., 2015. Genotypic distribution of hepatitis C virus in Thailand and southeast Asia. PLoS One 10 (5), e0126764.
White, P.A., Zhai, X., Carter, I., Zhao, Y., Rawlinson, W.D., 2000. Simplified hepatitis C virus genotyping by heteroduplex mobility analysis. J. Clin. Microbiol. 38 (2), 477–482.
Yang, R., Cong, X., Du, S., Fei, R., Rao, H., Wei, L., 2014. Performance comparison of the versant HCV genotype 2: 0 assay (LiPA) and the Abbott RealTime HCV genotype II assay for detecting hepatitis C virus genotype 6. J. Clin. Microbiol. 52, 3685–3692.